09/155327

PCT/AU 97/00199

Patent Office
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I, DAVID DANIEL CLARKE , ASSISTANT DIRECTOR PATENT SERVICES, hereby certify that the annexed are true copies of the Provisional specification and drawing(s) as filed on 27 March 1996 in connection with Application No. PN 8965 for a patent by THE WALTER AND ELIZA HALL INSTITUTE OF MEDICAL RESEARCH filed on 27 March 1996.

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DAVID DANIEL CLARKE
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THE WALTER AND ELIZA HALL INSTITUTE
OF MEDICAL RESEARCH

AUSTRALIA
Patents Act 1990

PROVISIONAL SPECIFICATION
for the invention entitled:

"THERAPEUTIC MOLECULES"

The invention is described in the following statement:

THERAPEUTIC MOLECULES

The present invention is broadly directed to therapeutic molecules capable of modulating apoptosis in mammalian cells. The therapeutic molecules of the present invention encompass genetic sequences and chemical entities capable of regulating expression of a novel gene belonging to the *bcl-2* family and which promotes cell survival. The present invention further extends to chemical entities capable of modulating activity and function of the translation product of said novel gene of the *bcl-2* family.

Bibliographic details of the publications numerically referred to in this specification are collected at the end of the description. Sequence Identity Numbers (SEQ ID NOs.) for the nucleotide and amino acid sequences referred to in the specification are defined following the bibliography.

Throughout this specification, unless the context requires otherwise, the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated element or integer or group of elements or integers but not the exclusion of any other element or integer or group of elements or integers.

The increasing sophistication of recombinant DNA technology is greatly facimiltating research and development in the medical and allied health fields. This technology is becoming particularly important in research into the treatment and diagnosis of both proliferative cell disorders such as cancers and sarcomas and in degenerative diseases such as some autoimmune conditions. There is a need to identify and characterise at the genetic level the elements involved in cell survival and physiological cell death (apoptosis).

Apoptosis is accomplished by a process that is conserved between organisms as diverse as nematodes and man. Positive and negative regulation of cell survival is essential for the proper development and differentiation of the embryo and for ensuring homeostasis with adult tissues. Cell survival can be promoted by the binding of growth factors to their receptors or by interaction of cellular adhesion molecules. A range of cytotoxic agents can counteract these signals and activate apoptosis, a process initially defined by specific morphologic criteria, such as chromatin condensation, cell compaction, membrane blebbing and, often, internucleosomal cleavage of DNA.

The biochemical details of the intracellular pathways governing cell survival and death remain largely undefined. However, several key regulators have emerged. The first to be discovered was Bcl-2, a 26 kD cytoplasmic protein encoded by the *bcl-2* gene translocated to the Igh locus in human follicular lymphoma. High levels of Bcl-2 greatly enhance the ability of cells to survive cytokine deprivation and a wide variety of other cytotoxic conditions, including DNA damage.

The mammalian genome contains other genes homologous to *bcl-2* but which differ in function. For example, *bcl-x* blocks apoptosis (Boise *et al,* 1993) whereas *bax* and *bak* inhibit the survival function of *bcl-2* and *bcl-x* (Oltvai *et al* , 1993; Chittenden *et al,* 1995; Farrow *et al.*, 1995; Kiefer *et al* , 1995). Due to the potential importance of cell apoptosis controlling genes in the treatment of cancers and sarcomas and in the treatment of degenerative disorders, there is a need to identify new genes homologous to *bcl-2* in structure and function.

In accordance with the present invention, the inventors have identified a novel gene designated herein "*bcl-w*". Gene transfer studies show that *bcl-w* enhances cell survival and belong to the *bcl-2* family of apoptosis-controlling genes. The identification of this new gene will lead to the generation of a range of therapeutic molecules capable of acting as either antagnosists or agonists of *bcl-w* expression or activity and will be useful in cancer or degenerative disease therapy.

Accordingly, one aspect of the present invention is directed to a nucleic acid molecule comprising a nucleotide sequence encoding or complementary to a sequence encoding the amino acid sequence set forth in SEQ ID NO. 1 or a derivative thereof.

The nucleic acid molecule according to this aspect of the present invention corresponds herein to "*bcl-w*". This gene has been determined by the inventors in accordance with the present invention to enhance cell survival. The product of the *bcl-w* gene is referred to as Bcl-w and is defined by the amino acid sequence set forth in SEQ ID NO. 1. Reference herein to "*bcl-w*" includes reference to derivatives thereof includes single or multiple nucleotide substitutions, deletions and/or additions. The gene is preferably from a mammal such as a human, livestock animal (sheep, pig, horse, donkey), laboratory test animal (eg. mouse, rat, rabbit, guinea pig), companion animal (eg. dog, cat) or captive wild animal (eg. fox, kangaroo).

The nucleic acid molecule of the present invention is preferably in isolated form or ligated to a vector, such as an expression vector. By "isolated" is meant a nucleic acid molecule having undergone at least one purification step and this is conveniently defined, for example, by a composition comprising at least about 10% subject nucleic acid molecule, preferably at least about 20%, more preferably at least about 30%, still more preferably at least about 40-50%, even still more preferably at least about 60-70%, yet even still more preferably 80-90% or greater of subject nucleic acid molecule relative to other components as determined by molecular weight, encoding activity, nucleotide sequence, base

composition or other convenient means. The nucleic acid molecule of the present invention may also be considered, in a preferred embodiment, to be biologically pure.

The nucleic acid molecule encoding *bcl-w* is preferably a sequence of deoxyribonucleic acids such as cDNA sequence or a genomic sequence. A genomic sequence may also comprise exons and introns. A genomic sequence may also include a promoter region or other regulatory region. In a particularly preferred embodiment, the nucleotide sequence corresponding to *bcl-w* is a cDNA sequence comprising a sequence of nucleotides as set forth in SEQ ID No: 2 or is a derivative thereof.

The term "derivative" as used herein includes portions, fragments, parts, homologues or analogues of the nucleic acid molecule or translation product. A derivative may also be a single or multiple nucleotide or amino acid substitution, deletion and/or addition. A derivative of the nucleic acid molecule of the present invention also includes nucleic acid molecules capable of hybridizing to the nucleotide sequence set forth in SEQ ID No: 2 under low stringency conditions.

The derivations of the nucleic acid molecule of the present invention include oligonucleotides, PCR primers, antisense molecules, molecules suitable for use in co-suppression and fusion nucleic acid molecules. Ribozymes are also contemplated by the present invention directed to *bcl-w*. The derivatives of the Bcl-w translation product of the present invention include fragments having particular epitopes or parts of the entire Bcl-w protein fused to peptides, polypeptides or other proteins.

As stated above, the nucleic acid molecule may be ligated to an expression vector capable of expression in a prokaryotic cell (e.g. *E.coli*) or a eukaryotic cell (e.g. yeast cells, fungal cells, insect cells, mammalian cells or plant cells).

The resulting expression product is Bcl-w having an amino acid sequence set forth in SEQ ID No: 1 or is a derivative thereof as defined above. Other derivatives include chemical analogues of Bcl-w. Analogues of Bcl-w contemplated herein include, but are not limited to, modification to side chains, incorporating of unnatural amino acids and/or their derivatives during peptide, polypeptide or protein synthesis and the use of crosslinkers and other methods which impose confirmational constraints on the proteinaceous molecule or their analogues.

Examples of side chain modifications contemplated by the present invention include modifications of amino groups such as by reductive alkylation by reaction with an aldehyde followed by reduction with $NaBH_4$; amidination with methylacetimidate; acylation with acetic anhydride; carbamoylation of amino groups with cyanate; trinitrobenzylation of amino groups with 2, 4, 6-trinitrobenzene sulphonic acid (TNBS); acylation of amino groups with succinic anhydride and tetrahydrophthalic anhydride; and pyridoxylation of lysine with pyridoxal-5-phosphate followed by reduction with $NaBH_4$.

The guanidine group of arginine residues may be modified by the formation of heterocyclic condensation products with reagents such as 2,3-butanedione, phenylglyoxal and glyoxal.

The carboxyl group may be modified by carbodiimide activation *via* O-acylisourea formation followed by subsequent derivitisation, for example, to a corresponding amide.

Sulphydryl groups may be modified by methods such as carboxymethylation with iodoacetic acid or iodoacetamide; performic acid oxidation to cysteic acid; formation of a mixed disulphides with other thiol compounds; reaction with maleimide, maleic anhydride or other substituted maleimide; formation of mercurial derivatives using 4-chloromercuribenzoate, 4-chloromercuriphenylsulphonic acid, phenylmercury chloride, 2-chloromercuri-4-nitrophenol and other mercurials; carbamoylation with cyanate at alkaline pH.

Tryptophan residues may be modified by, for example, oxidation with N-bromosuccinimide or alkylation of the indole ring with 2-hydroxy-5-nitrobenzyl bromide or sulphenyl halides. Tyrosine residues on the other hand, may be altered by nitration with tetranitromethane to form a 3-nitrotyrosine derivative.

Modification of the imidazole ring of a histidine residue may be accomplished by alkylation with iodoacetic acid derivatives or N-carbethoxylation with diethylpyrocarbonate.

Examples of incorporating unnatural amino acids and derivatives during peptide synthesis include, but are not limited to, use of norleucine, 4-amino butyric acid, 4-amino-3-hydroxy-5-phenylpentanoic acid, 6-aminohexanoic acid, t-butylglycine, norvaline, phenylglycine, ornithine, sarcosine, 4-amino-3-hydroxy-6-methylheptanoic acid, 2-thienyl alanine and/or D-isomers of amino acids. A list of unnatural amino acid, contemplated herein is shown in Table 3.

Crosslinkers can be used, for example, to stabilise 3D conformations, using homo-bifunctional crosslinkers such as the bifunctional imido esters having $(CH_2)_n$ spacer groups with n=1 to n=6, glutaraldehyde, N-hydroxysuccinimide esters and hetero-bifunctional reagents which usually contain an amino-reactive moiety such as N-hydroxysuccinimide and another group specific-reactive moiety such as maleimido or dithio moiety (SH) or carbodiimide (COOH). In addition, peptides can be conformationally constrained by, for example, incorporation of C_α and N_α-methylamino acids, introduction of double bonds between C_α and C_β atoms of amino acids and the formation of cyclic peptides or analogues by introducing covalent bonds such as forming an amide bond between the N and C termini, between two side chains or between a side chain and the N or C terminus.

The identification of *bcl-w* permits the generation of a range of therapeutic molecules capable of modulating expression of *bcl-w* or modulating the activity of Bcl-2. Modulators contemplated by the present invention includes agonists and antagonists of *bcl-w* expression. Antagonists of *bcl-w* expression include antisense molecules, ribozymes and co-suppression molecules. Agonists include molecules which increase promoter ability or interfere with negative regulatory mechanisms. Agonists of Bcl-w include molecules which overcome any negative regulatory mechanism. Antagonists of Bcl-w include antibodies and inhibitor peptide fragments.

TABLE 3

Non-conventional amino acid	Code	Non-conventional amino acid	Code
α-aminobutyric acid	Abu	L-N-methylalanine	Nmala
α-amino-α-methylbutyrate	Mgabu	L-N-methylarginine	Nmarg
aminocyclopropane-carboxylate	Cpro	L-N-methylasparagine	Nmasn
		L-N-methylaspartic acid	Nmasp
aminoisobutyric acid	Aib	L-N-methylcysteine	Nmcys
aminonorbornyl-carboxylate	Norb	L-N-methylglutamine	Nmgln
		L-N-methylglutamic acid	Nmglu
cyclohexylalanine		Chexa L-N-methylhistidine	Nmhis
cyclopentylalanine	Cpen	L-N-methylisolleucine	Nmile
D-alanine	Dal	L-N-methylleucine	Nmleu
D-arginine	Darg	L-N-methyllysine	Nmlys
D-aspartic acid	Dasp	L-N-methylmethionine	Nmmet
D-cysteine	Dcys	L-N-methylnorleucine	Nmnle
D-glutamine	Dgln	L-N-methylnorvaline	Nmnva

	D-glutamic acid	Dglu	L-N-methylornithine	Nmorn
	D-histidine	Dhis	L-N-methylphenylalanine	Nmphe
	D-isoleucine	Dile	L-N-methylproline	Nmpro
	D-leucine	Dleu	L-N-methylserine	Nmser
5	D-lysine	Dlys	L-N-methylthreonine	Nmthr
	D-methionine	Dmet	L-N-methyltryptophan	Nmtrp
	D-ornithine	Dorn	L-N-methyltyrosine	Nmtyr
	D-phenylalanine	Dphe	L-N-methylvaline	Nmval
	D-proline	Dpro	L-N-methylethylglycine	Nmetg
10	D-serine	Dser	L-N-methyl-t-butylglycine	Nmtbug
	D-threonine	Dthr	L-norleucine	Nle
	D-tryptophan	Dtrp	L-norvaline	Nva
	D-tyrosine	Dtyr	α-methyl-aminoisobutyrate	Maib
	D-valine	Dval	α-methyl-γ-aminobutyrate	Mgabu
15	D-α-methylalanine	Dmala	α-methylcyclohexylalanine	Mchexa
	D-α-methylarginine	Dmarg	α-methylcylcopentylalanine	Mcpen
	D-α-methylasparagine	Dmasn	α-methyl-α-napthylalanine	Manap
	D-α-methylaspartate	Dmasp	α-methylpenicillamine	Mpen
	D-α-methylcysteine	Dmcys	N-(4-aminobutyl)glycine	Nglu
20	D-α-methylglutamine	Dmgln	N-(2-aminoethyl)glycine	Naeg
	D-α-methylhistidine	Dmhis	N-(3-aminopropyl)glycine	Norn
	D-α-methylisoleucine	Dmile	N-amino-α-methylbutyrate	Nmaabu
	D-α-methylleucine	Dmleu	α-napthylalanine	Anap
	D-α-methyllysine	Dmlys	N-benzylglycine	Nphe
25	D-α-methylmethionine	Dmmet	N-(2-carbamylethyl)glycine	Ngln
	D-α-methylornithine	Dmorn	N-(carbamylmethyl)glycine	Nasn
	D-α-methylphenylalanine	Dmphe	N-(2-carboxyethyl)glycine	Nglu
	D-α-methylproline	Dmpro	N-(carboxymethyl)glycine	Nasp
	D-α-methylserine	Dmser	N-cyclobutylglycine	Ncbut
30	D-α-methylthreonine	Dmthr	N-cycloheptylglycine	Nchep

	D-α-methyltryptophan	Dmtrp	N-cyclohexylglycine	Nchex
	D-α-methyltyrosine	Dmty	N-cyclodecylglycine	Ncdec
	D-α-methylvaline	Dmval	N-cylcododecylglycine	Ncdod
	D-N-methylalanine	Dnmala	N-cyclooctylglycine	Ncoct
5	D-N-methylarginine	Dnmarg	N-cyclopropylglycine	Ncpro
	D-N-methylasparagine	Dnmasn	N-cycloundecylglycine	Ncund
	D-N-methylaspartate	Dnmasp	N-(2,2-diphenylethyl)glycine	Nbhm
	D-N-methylcysteine	Dnmcys	N-(3,3-diphenylpropyl)glycine	Nbhe
	D-N-methylglutamine	Dnmgln	N-(3-guanidinopropyl)glycine	Narg
10	D-N-methylglutamate	Dnmglu	N-(1-hydroxyethyl)glycine	Nthr
	D-N-methylhistidine	Dnmhis	N-(hydroxyethyl))glycine	Nser
	D-N-methylisoleucine	Dnmile	N-(imidazolylethyl))glycine	Nhis
	D-N-methylleucine	Dnmleu	N-(3-indolylyethyl)glycine	Nhtrp
	D-N-methyllysine	Dnmlys	N-methyl-γ-aminobutyrate	Nmgabu
15	N-methylcyclohexylalanine	Nmchexa	D-N-methylmethionine	Dnmmet
	D-N-methylornithine	Dnmorn	N-methylcyclopentylalanine	Nmcpen
	N-methylglycine	Nala	D-N-methylphenylalanine	Dnmphe
	N-methylaminoisobutyrate	Nmaib	D-N-methylproline	Dnmpro
	N-(1-methylpropyl)glycine	Nile	D-N-methylserine	Dnmser
20	N-(2-methylpropyl)glycine	Nleu	D-N-methylthreonine	Dnmthr
	D-N-methyltryptophan	Dnmtrp	N-(1-methylethyl)glycine	Nval
	D-N-methyltyrosine	Dnmtyr	N-methyla-napthylalanine	Nmanap
	D-N-methylvaline	Dnmval	N-methylpenicillamine	Nmpen
	γ-aminobutyric acid	Gabu	N-(*p*-hydroxyphenyl)glycine	Nhtyr
25	L-*t*-butylglycine	Tbug	N-(thiomethyl)glycine	Ncys
	L-ethylglycine	Etg	penicillamine	Pen
	L-homophenylalanine	Hphe	L-α-methylalanine	Mala
	L-α-methylarginine	Marg	L-α-methylasparagine	Masn
	L-α-methylaspartate	Masp	L-α-methyl-*t*-butylglycine	Mtbug
30	L-α-methylcysteine	Mcys	L-methylethylglycine	Metg

L-α-methylglutamine	Mgln	L-α-methylglutamate	Mglu
L-α-methylhistidine	Mhis	L-α-methylhomophenylalanine	Mhphe
L-α-methylisoleucine	Mile	N-(2-methylthioethyl)glycine	Nmet
L-α-methylleucine	Mleu	L-α-methyllysine	Mlys
L-α-methylmethionine	Mmet	L-α-methylnorleucine	Mnle
L-α-methylnorvaline	Mnva	L-α-methylornithine	Morn
L-α-methylphenylalanine	Mphe	L-α-methylproline	Mpro
L-α-methylserine	Mser	L-α-methylthreonine	Mthr
L-α-methyltryptophan	Mtrp	L-α-methyltyrosine	Mtyr
L-α-methylvaline	Mval	L-N-methylhomophenylalanine	Nmhphe
N-(N-(2,2-diphenylethyl) carbamylmethyl)glycine	Nnbhm	N-(N-(3,3-diphenylpropyl) carbamylmethyl)glycine	Nnbhe
1-carboxy-1-(2,2-diphenyl-ethylamino)cyclopropane	Nmbc		

The present invention contemplates, therefore, a method for modulating expression of *bcl-w* in a mammal, said method comprising contacting the *bcl-w* gene with an effective amount of a modulator of *bcl-w* expression for a time and under conditions sufficient to up-regulate or down-regulate or otherwise modulate expression of *bcl-w*. For example, a nucleic acid molecule encoding Bcl-w or a derivative thereof may be introduced into a cell to enhance the ability of that cell to survive, conversely, *bcl-w* antisense sequences such as oligonucleotides may be introduced to decrease the survival capacity of any cell expressing the endogenous *bcl-w* gene.

Another aspect of the present invention contemplates a method of modulating activity of Bcl-w in a mammal, said method comprising administering to said mammal a modulating effective amount of a molecule for a time and under conditions sufficient to increase or decrease Bcl-w activity. The molecule may be a proteinaceous molecule or a chemical

entity and may also be a derivative of Bcl-w or its receptor.

Increased *bcl-w* expression or *Bcl-w* activity is important in conditions of cell degeneracy such as under cytotoxic conditions during, for example, γ-irradiation and chemotherapy. Decreased *bcl-w* expression or Bcl-w activity may be important, for example, in selective cancer therapy or prophylaxis of target cells, as well as for the treatment or prophylaxis of conditions such as stroke, Alzheimer's disease, ataxia telangiectasia, Bloom's syndrome and progeria.

Accordingly, the present invention contemplates a pharmaceutical composition comprising a modulator of *bcl-w* expression or Bcl-w activity and one or more pharmaceutically acceptable carriers and/or diluents.

The pharmaceutical forms suitable for injectable use include sterile aqueous solutions (where water soluble) or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersion. In all cases the form must be sterile and must be fluid to the extent that easy syringability exists. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms such as bacteria and fungi. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol and liquid polyethylene glycol, and the like), suitable mixtures thereof, and vegetable oils. The proper fluidity can be maintained, for example, by the use of a coating such as licithin, by the maintenance of the required particle size in the case of dispersion and by the use of superfactants. The preventions of the action of microorganisms can be brought about by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, thirmerosal and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars or sodium chloride. Prolonged absorption of the injectable compositions can be brought about by the use in the compositions of agents delaying absorption, for example, aluminum monostearate and gelatin.

Sterile injectable solutions are prepared by incorporating the active compounds in the required amount in the appropriate solvent with various of the other ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the various sterilized active ingredient into a sterile vehicle which contains the basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum drying and the freeze-drying technique which yield a powder of the active ingredient plus any additional desired ingredient from previously sterile-filtered solution thereof.

When *blc-w* and Bcl-w modulators are suitably protected they may be orally administered, for example, with an inert diluent or with an assimilable edible carrier, or it may be enclosed in hard or soft shell gelatin capsule, or it may be compressed into tablets, or it may be incorporated directly with the food of the diet. For oral therapeutic administration, the active compound may be incorporated with excipients and used in the form of ingestible tablets, buccal tablets, troches, capsules, elixirs, suspensions, syrups, wafers, and the like. Such compositions and preparations should contain at least 1% by weight of active compound. The percentage of the compositions and preparations may, of course, be varied and may conveniently be between about 5 to about 80% of the weight of the unit. The amount of active compound in such therapeutically useful compositions in such that a suitable dosage will be obtained. Preferred compositions or preparations according to the present invention are prepared so that an oral dosage unit form contains between about 0.1 ug and 2000 mg of active compound.

The tablets, troches, pills, capsules and the like may also contain the following: A binder such as gum tragacanth, acacia, corn starch or gelatin; excipients such as dicalcium phosphate; a disintegrating agent such as corn starch, potato starch, alginic acid and the like; a lubricant such as magnesium stearate; and a sweetening agent such a sucrose, lactose or saccharin may be added or a flavouring agent such as peppermint, oil of

wintergreen, or cherry flavouring. When the dosage unit form is a capsule, it may contain, in addition to materials of the above type, a liquid carrier. Various other materials may be present as coatings or to otherwise modify the physical form of the dosage unit. For instance, tablets, pills, or capsules may be coated with shellac, sugar or both. A syrup or elixir may contain the active compound, sucrose as a sweetening agent, methyl and propylparabens as preservatives, a dye and flavouring such as cherry or orange flavour. Of course, any material used in preparing any dosage unit form should be pharmaceutically pure and substantially non-toxic in the amounts employed. In addition, the active compound may be incorporated into sustained-release preparations and formulations.

Pharmaceutically acceptable carriers and/or diluents include any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents and the like. The use of such media and agents for pharmaceutical active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active ingredient, use thereof in the therapeutic compositions is contemplated. Supplementary active ingredients can also be incorporated into the compositions.

It is especially advantageous to formulate parenteral compositions in dosage unit form for ease of administration and uniformity of dosage. Dosage unit form as used herein refers to physically discrete units suited as unitary dosages for the mammalian subjects to be treated; each unit containing a predetermined quantity of active material calculated to produce the desired therapeutic effect in association with the required pharmaceutical carrier. The specification for the novel dosage unit forms of the invention are dictated by and directly dependent on (a) the unique characteristics of the active material and the particular therapeutic effect to be achieved, and (b) the limitations inherent in the art of compounding such an active material for the treatment of disease in living subjects having a diseased condition in which bodily health is impaired as herein disclosed in detail.

The principal active ingredient is compounded for convenient and effective administration in effective amounts with a suitable pharmaceutically acceptable carrier in dosage unit form as hereinbefore disclosed. A unit dosage form can, for example, contain the principal active compound in amounts ranging from 0.5 μg to about 2000 mg. Expressed in proportions, the active compound is generally present in from about 0.5 μg to about 2000 mg/ml of carrier. In the case of compositions containing supplementary active ingredients, the dosages are determined by reference to the usual dose and manner of administration of the said ingredients.

The pharmaceutical composition may also comprise genetic molecules such as a vector capable of transfecting target cells where the vector carries a nucleic acid molecule capable of modulating *bcl-w* expression or Bcl-w activity. The vector may, for example, be a viral vector.

Conditions requiring modulation of physiological cell death include enhancing survival of cells in patients with neurodegenerative diseases, myocardial infarction, muscular degenerative disease, hypoxia, ischaemia, HIV infection or for prolonging the survival of cells being transplanted for treatment of disease. Alternatively, the antisense sequence could be used, for example, to reduce the survival capacity of tumour cells or autoreactive lymphocytes. The sense sequence may also be used for modifying *in vitro* behaviour of cells, for example, as part of a protocol to develop novel lines from cell types having unidentified growth factor requirements; for facilitating isolation of hybridoma cells producing monoclonal antibodies, as described below; and for enhancing survival of cells from primary explants while they are being genetically modified.

Still another aspect of the present invention is directed to antibodies to Bcl-w and its derivatives. Such antibodies may be monoclonal or polyclonal and may be selected from naturally occurring antibodies to Bcl-w or may be specifically raised to Bcl-w or derivatives thereof. In the case of the latter, Bcl-w or its derivatives may first need to be associated with a carrier molecule. The antibodies and/or recombinant Bcl-w or its derivatives of the present invention are particularly useful as therapeutic or diagnostic agents.

For example, Bcl-w and its derivatives can be used to screen for naturally occurring antibodies to Bcl-w. These may occur, for example in some autoimmune diseases. Alternatively, specific antibodies can be used to screen for Bcl-w. Techniques for such assays are well known in the art and include, for example, sandwich assays and ELIZA.

Antibodies to Bcl-w of the present invention may be monoclonal or polyclonal and may be selected from naturally occurring antibodies to the Bcl-w or may be specifically raised to the Bcl-w or its derivatives. In the case of the latter, the Bcl-w protein may need first to be associated with a carrier molecule. Alternatively, fragments of antibodies may be used such as Fab fragments. Furthermore, the present invention extends to recombinant and synthetic antibodies and to antibody hybrids. A "synthetic antibody" is considered herein to include fragments and hybrids of antibodies. The antibodies of this aspect of the present invention are particularly useful for immunotherapy and may also be used as a diagnostic tool for assessing apoptosis or monitoring the program of a therapeutic regima.

For example, specific antibodies can be used to screen for Bcl-w proteins. The latter would be important, for example, as a means for screening for levels of Bcl-w in a cell extract or other biological fluid or purifying Bcl-w made by recombinant means from culture supernatant fluid. Techniques for the assays contemplated herein are known in the art and include, for example, sandwich assays and ELISA.

It is within the scope of this invention to include any second antibodies (monoclonal, polyclonal or fragments of antibodies) directed to the first mentioned antibodies discussed above. Both the first and second antibodies may be used in detection assays or a first antibody may be used with a commercially available anti-immunoglobulin antibody. An antibody as contemplated herein includes any antibody specific to any region of Bcl-w.

Both polyclonal and monoclonal antibodies are obtainable by immunization with the enzyme or protein and either type is utilizable for immunoassays. The methods of obtaining both types of sera are well known in the art. Polyclonal sera are less preferred but are relatively easily prepared by injection of a suitable laboratory animal with an effective amount of Bcl-w, or antigenic parts thereof, collecting serum from the animal, and isolating specific sera by any of the known immunoadsorbent techniques. Although antibodies produced by this method are utilizable in virtually any type of immunoassay, they are generally less favoured because of the potential heterogeneity of the product.

The use of monoclonal antibodies in an immunoassay is particularly preferred because of the ability to produce them in large quantities and the homogeneity of the product. The preparation of hybridoma cell lines for monoclonal antibody production derived by fusing an immortal cell line and lymphocytes sensitized against the immunogenic preparation can be done by techniques which are well known to those who are skilled in the art. (See, for example Douillard and Hoffman, Basic Facts about Hybridomas, in *Compendium of Immunology* Vol II, ed. by Schwartz, 1981; Kohler and Milstein, *Nature 256*: 495-499, 1975; *European Journal of Immunology 6*: 511-519, 1976).

Another aspect of the present invention contemplates a method for detecting Bcl-w in a biological sample from a subject said method comprising contacting said biological sample with an antibody specific for Bcl-w or its derivatives or homologues for a time and under conditions sufficient for an antibody-Bcl-w complex to form, and then detecting said complex.

The presence of Bcl-w may be accomplished in a number of ways such as by Western blotting and ELISA procedures. A wide range of immunoassay techniques are available as can be seen by reference to US Patent Nos. 4,016,043, 4, 424,279 and 4,018,653. These, of course, includes both single-site and two-site or "sandwich" assays of the non-competitive types, as well as in the traditional competitive binding assays. These assays also include direct binding of a labelled antibody to a target.

Sandwich assays are among the most useful and commonly used assays and are favoured for use in the present invention. A number of variations of the sandwich assay technique exist, and all are intended to be encompassed by the present invention. Briefly, in a typical forward assay, an unlabelled antibody is immobilized on a solid substrate and the sample to be tested brought into contact with the bound molecule. After a suitable period of incubation, for a period of time sufficient to allow formation of an antibody-antigen complex, a second antibody specific to the antigen, labelled with a reporter molecule capable of producing a detectable signal is then added and incubated, allowing time sufficient for the formation of another complex of antibody-antigen-labelled antibody. Any unreacted material is washed away, and the presence of the antigen is determined by observation of a signal produced by the reporter molecule. The results may either be qualitative, by simple observation of the visible signal, or may be quantitated by comparing with a control sample containing known amounts of hapten. Variations on the forward assay include a simultaneous assay, in which both sample and labelled antibody are added simultaneously to the bound antibody. These techniques are well known to those skilled in the art, including any minor variations as will be readily apparent. In accordance with the present invention the sample is one which might contain Bcl-w including cell extract,

tissue biopsy or possibly serum, saliva, mucosal secretions, lymph, tissue fluid and respiratory fluid. The sample is, therefore, generally a biological sample comprising biological fluid but also extends to fermentation fluid and supernatant fluid such as from a cell culture.

In the typical forward sandwich assay, a first antibody having specificity for the Bcl-w or antigenic parts thereof, is either covalently or passively bound to a solid surface. The solid surface is typically glass or a polymer, the most commonly used polymers being cellulose, polyacrylamide, nylon, polystyrene, polyvinyl chloride or polypropylene. The solid supports may be in the form of tubes, beads, discs of microplates, or any other surface suitable for conducting an immunoassay. The binding processes are well-known in the art and generally consist of cross-linking covalently binding or physically adsorbing, the polymer-antibody complex is washed in preparation for the test sample. An aliquot of the sample to be tested is then added to the solid phase complex and incubated for a period of time sufficient (e.g. 2-40 minutes) and under suitable conditions (e.g. 25°C) to allow binding of any subunit present in the antibody. Following the incubation period, the antibody subunit solid phase is washed and dried and incubated with a second antibody specific for a portion of the hapten. The second antibody is linked to a reporter molecule which is used to indicate the binding of the second antibody to the hapten.

An alternative method involves immobilizing the target molecules in the biological sample and then exposing the immobilized target to specific antibody which may or may not be labelled with a reporter molecule. Depending on the amount of target and the strength of the reporter molecule signal, a bound target may be detectable by direct labelling with the antibody. Alternatively, a second labelled antibody, specific to the first antibody is exposed to the target-first antibody complex to form a target-first antibody-second antibody tertiary complex. The complex is detected by the signal emitted by the reporter molecule.

By "reporter molecule" as used in the present specification, is meant a molecule which, by its chemical nature, provides an analytically identifiable signal which allows the detection of antigen-bound antibody. Detection may be either qualitative or quantitative. The most commonly used reporter molecules in this type of assay are either enzymes, fluorophores or radionuclide containing molecules (i.e. radioisotopes) and chemiluminescent molecules.

In the case of an enzyme immunoassay, an enzyme is conjugated to the second antibody, generally by means of glutaraldehyde or periodate. As will be readily recognized, however, a wide variety of different conjugation techniques exist, which are readily available to the skilled artisan. Commonly used enzymes include horseradish peroxidase, glucose oxidase, beta-galactosidase and alkaline phosphatase, amongst others. The substrates to be used with the specific enzymes are generally chosen for the production, upon hydrolysis by the corresponding enzyme, of a detectable color change. Examples of suitable enzymes include alkaline phosphatase and peroxidase. It is also possible to employ fluorogenic substrates, which yield a fluorescent product rather than the chromogenic substrates noted above. In all cases, the enzyme-labelled antibody is added to the first antibody hapten complex, allowed to bind, and then the excess reagent is washed away. A solution containing the appropriate substrate is then added to the complex of antibody-antigen-antibody. The substrate will react with the enzyme linked to the second antibody, giving a qualitative visual signal, which may be further quantitated, usually spectrophotometrically, to give an indication of the amount of hapten which was present in the sample. "Reporter molecule" also extends to use of cell agglutination or inhibition of agglutination such as red blood cells on latex beads, and the like.

Alternately, fluorescent compounds, such as fluorecein and rhodamine, may be chemically coupled to antibodies without altering their binding capacity. When activated by illumination with light of a particular wavelength, the fluorochrome-labelled antibody adsorbs the light energy, inducing a state to excitability in the molecule, followed by emission of the light at a characteristic color visually detectable with a light microscope.

As in the EIA, the fluorescent labelled antibody is allowed to bind to the first antibody-hapten complex. After washing off the unbound reagent, the remaining tertiary complex is then exposed to the light of the appropriate wavelength the fluorescence observed indicates the presence of the hapten of interest. Immunofluorescene and EIA techniques are both very well established in the art and are particularly preferred for the present method. However, other reporter molecules, such as radioisotope, chemiluminescent or bioluminescent molecules, may also be employed.

The present invention also contemplates genetic assays such as involving PCR analysis to detect *bcl-w* or its derivatives.

The present invention is further described by reference to the following non-limiting figures and examples.

In the Figures:

Figure 1 is a representation showing predicted amino acid sequences encoded by murine *bcl-w* cDNAs and chimaeric cDNAs corresponding to transcripts spliced from exon 3 of the *bcl-w* gene to an exon of the adjacent *rox* gene. Boxes highlight the regions of highest homology within the Bcl-2 family, denoted S1, S2 and S3 (Cory, 1995). The arrowhead marks the position corresponding to an intron within the gene. Two residues that differ in human Bcl-w are indicated above the mouse sequence. Not all of the *rox* cDNA sequence was determined in both orientations.

Figure 2 is a diagrammatic representation showing the structure of the genomic *bcl-w* locus and derivation of the *bcl-w* and *bcl-w/rox* cDNAs. Overlapping genomic fragments encompassing a 22 kb region were cloned, only one of which (*a*) is shown. Fragments *b* to *f* are subclones of fragment *a*. Exons are denoted as boxes, with non-coding regions open, the coding region of the *bcl-w* gene filled and that of the *rox* gene (see text) stippled. Two types of 5'-end were found for each class of mRNA, suggestive of alternative

promoters and/or splicing. The first 815 residues of the 3' untranslated region of *bcl-w* correspond precisely to those in genomic exon 4; the region not yet sequenced is indicated as a broken line. Restriction mapping suggests the 3' untranslated region of *bcl-w* contains at least one more intron. The location of the remainder of the *rox* gene is not known.

Figure 3 is a photographic representation showing expression of *bcl-w* RNA in haemopoietic cell lines. Polyadenylated RNA prepared from the indicated macrophage (mϕ), myeloid, and T and B lymphoid lines (see Materials and methods), was fractionated by electrophoresis, transferred to nitrocellulose filters and hybridised with a *bcl-w* cDNA probe. Probes from the coding region and the *bcl-w* 3' untranslated region gave identical results.

Figure 4 shows the expression of Bcl-w protein. (A) Expression of FLAG-Bcl-w within a clone (D3B5) of FDC-P1 cells transfected with the FLAG-*bcl-w* PGKpuro expression vector. Transfectants (filled) and parental cells (open) were stained with anti-FLAG monoclonal antibody and analysed by flow cytometry (see Materials and methods). (B) Immunoblots revealing epitope-tagged survival proteins. Lysates of FDC-P1 cells and FDC-P1 cells expressing FLAG-tagged mouse Bcl-w (clone D3B5), human Bcl-x_L or human Bcl-2 were passed over an anti-FLAG affinity gel (Kodak), eluted with FLAG peptide, fractionated by electrophoresis and then analysed with anti-FLAG antibody. (C) Immunoblots with polyclonal rabbit anti-Bcl-w antiserum on cell lysates fractionated by SDS-polyacrylamide gel electrophoresis. In (B) and (C), the stained proteins were visualised by enhanced chemiluminescence (Amersham). WEHI-112.1 and EL4.1 are T lymphoma lines (Harris *et al.*, 1973) and J774 is a macrophage line (Ralph *et al.*, 1975). An additional protein of ~18 kD was also detected by the antiserum, apparently by fortuitous cross-reaction. The molecular weights of markers (Bio-Rad) are given in kD.

Figure 5 is a graphical representation showing that Bcl-w inhibits apoptosis induced by several but not all cytotoxic agents. FDC-P1 cells, which require IL-3 for survival and proliferation (Dexter *et al.*, 1980), were either (A, left panel) washed three times in

medium lacking IL-3 or (A, right panel) irradiated (10 Gy) and then cultured in medium lacking (A, left panel) or containing IL-3 (A, right panel). B6.2.16BW2 T hybridoma cells (Teh *et al.*, 1989) were either cultured in medium containing 1 μM dexamethasone (B, left panel) or irradiated (10Gy) (B, right panel). CH1 B lymphoma cells (Lynes *et al.*, 1978) were either cultured in the presence of 0.1 μg/ml Jo2 anti-mouse CD95 antibody (Ogasawara *et al.*, 1993) (C, left panel) or irradiated (10 Gy) (C, right panel). Cultures were initiated at 2.5 x 10^5 cells/ml and viability determined by staining with 0.4% w/v eosin on the indicated days.

Figure 6 is a diagrammatic representation showing that *Bcl-w* maps in the central region of mouse chromosome 14. The segregation patterns of *bcl-w* and flanking genes in 134 backcross animals typed for all loci are shown at the top. Each column represents the haplotype inherited from the (C57BL/6J x *M. spretus*) F_1 parent; shaded boxes represent the C57BL/6J allele and open boxes the *M. spretus* allele. The number of offspring inheriting each type of chromosome is listed below each column. A partial chromosome 14 linkage map showing the location of *bcl-w* in relation to linked genes is shown at the bottom. Recombination distances between loci in centiMorgans are shown to the left of the chromosome and the positions of loci in human chromosomes, where known, are shown to the right. References for the human map positions of loci cited in this study can be obtained from GDB (Genome Data Base), a database of human linkage information maintained by The William H. Welch Medical Library of The Johns Hopkins University (Baltimore, MD).

Figure 7 is a photographic representation showing localisation of *bcl-w* on human chromosome 14. Partial metaphase showing FISH with the *bcl-w* intronic probe. (A) Normal male chromosomes stained with propidium iodide. Hybridisation sites on chromosome 14 are indicated by an arrow. (B) the same metaphase as in (A) stained with DAPI for chromosome identification.

Figure 8 is a representation of a comparison of survival and anti-survival Bcl-2 sub-families. Human Bcl-2, Bcl-x_L, Bcl-w, Bax and Bak amino acid sequences were aligned by the Wisconsin PILEUP program. The most conserved portion of the Ced 9 sequence and a short conserved segment in Bik are also shown. Gaps made in individual sequences to optimise alignment are indicated by dots. Residues identical or very similar (L ~ M; E ~ D; K ~ R; V~I) in the survival-promoting proteins Bcl-2, Bcl-x_L and Bcl-w are shown on a black background, as are also those identical or very similar in all the Bcl-2 homologues. A grey background indicates residues shared by Bak and Bax but not present in the survival proteins. Homology regions S1, S2 and S3 (Cory, 1995) and the hydrophobic C-terminal segment are boxed, while the BH1, BH2, BH3 and NH1 regions defined by others (Yin *et al.*, 1994; Subramanian *et al.*, 1995) are overlined. Filled arrowheads indicate conserved residues specific to the survival proteins; open arrowheads, those specific to anti-survival proteins. An unbroken arrow indicates the position of the splice site common to all the proteins; a broken arrow, the position of the alternative 5' splice that creates the smaller Bcl-x protein and a wavy line a conserved C-terminal motif.

Figure 9 is a representation of the coding region of (A) human and (B) murine *bcl-w*.

Single and triple letter abbreviations for amino acid residues are used in the subject specification, as defined in Table 2.

TABLE 2

AMINO ACID ABBREVIATIONS

Amino Acid	Three-letter Abbreviation	One-letter Symbol
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic acid	Asp	D
Cysteine	Cys	C
Glutamine	Gln	Q
Glutamic acid	Glu	E
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V
Any residue	Xaa	X

EXAMPLE 1
PCR CLONING

PCR cloning. Based on the strong homology between Bcl-2, Bcl-x_L and Bax, degenerate PCR primers were designed within the S2 and S3 regions (see Figure 8), using inosine at totally degenerate positions. To facilitate cloning, Xba I and Eco RI restriction sites were incorporated at the ends. The 5' primer was 5'GCTCTAG AAC TGG GGI (AC)GI (AG)TI GTI GCC TT(CT) TT3' [SEQ ID NO 1], corresponding to Xba I - NWGR(IV)VAFF [SEQ ID NO:2], and the 3' primer was 5'GGAAT TC CCA GCC ICC IT(GT) ITC TTG GAT CCA 3' [SEQ ID NO:3], corresponding to WIQ(DE)(NQ)GGW - Eco RI [SEQ ID NO:4]. Polyadenylated RNA templates (1 μg) for reverse transcription came from the mouse macrophage cell line J774 and d18 mouse brain. The RNA was ethanol precipitated, dried, resuspended in 10 μl of water, heated at 65°C for 10 min and chilled on ice. It was then reverse transcribed in a 20 μl reaction containing 50 mM TrisHCl (pH8.3 at 25°C), 75 mM KCl, 3 mM $MgCl_2$, 10 mM dithiothreitol, 0.5 mM dNTPs, 2 μl random hexamer primers (Amersham First Strand cDNA Synthesis System) and 200 U Superscript II™ reverse transcriptase (GIBCO), at 48°C for 60 min. For the PCR reaction, 1 μl of this reaction mixture was added to 49 μl of a cocktail consisting of 50 mM KCl, 10 mM TrisHCl (pH 9.0 at 25°C), 0.1% v/v Triton X-100, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 10% v/v glycerol, 0.05% w/v gelatine, 0.3 μg of each primer and 2.5 U Taq DNA polymerase. This mixture was denatured at 94°C for 3 min, then subjected to 5 cycles comprising 1 min at 94°C , 2 min at 37°C, ramping at 0.3°C/sec to 72°C followed by 1 min at 72°C. The thermal profile for the following 35 cycles was 1 min at 94°C, 2 min at 42°C, 1 min at 72°C. Finally, the mixture was incubated at 72°C for 5 min. The PCR products were fractionated by gel electrophoresis and DNA fragments of the expected size (159 bp) were extracted from the gel, restricted with Eco RI and Xba I and subcloned into Eco RI/Xba I-digested pBluescript II SK(+). The resulting clones were sequenced using a single base (T) reaction using the fmol™ Sequencing System (Promega) and the manufacturer's protocol. Complete sequence analysis was then performed on a representative clone for each unique T-track

pattern.

EXAMPLE 2

INTERSPECIFIC MOUSE BACKCROSS MAPPING

Interspecific backcross progeny were generated by mating (C57BL/6J ♀ *M. spretus*)F_1 females and C57BL/6J males as described (Copeland and Jenkins, 1991). A total of 205 N_2 mice were used to map the *Bcl-w* locus. DNA isolation, restriction enzyme digestion, agarose gel electrophoresis, Southern blot transfer and hybridisation were performed essentially as described (Jenkins *et al.*, 1982). The probe, a 2.6 kb *Eco*RI/*Not*I fragment of mouse cDNA, was labelled with (α^{32}P) dCTP using a random primed labelling kit (Stratagene); washing was done to a final stringency of 1.0 x SSCP, 0.1% w/v SDS, 65°C. A fragment of 3.8 kb was detected in BamHI digests of C57BL/6J DNA and 7.8 kb in *M. spretus* DNA. Their distribution was followed in backcross mice. The probes and RFLPs for the loci linked to *bcl-w*, including surfactant associated protein 1 (*Sftp1*), T cell receptor alpha chain (*Tcrα*), and gap junction membrane channel protein alpha-3 (*Gjα3*), have been described previously (Haefliger *et al.*, 1992; Moore *et al.*, 1992). Recombination distances were calculated as described (Green, 1981) using the computer program SPRETUS MADNESS. Gene order was determined by minimising the number of recombination events required to explain the allele distribution patterns.

EXAMPLE 3

FLUORESCENCE *IN SITU* HYBRIDISATION (*FISH*)

cDNA and intron probes were nick-translated with biotin-14-dATP and hybridised *in situ* at a final concentration of 20 ng/ml to normal male metaphases. The FISH method was modified from that previously described (Callen *et al.*, 1990) in that chromosomes were stained before analysis with both propidium iodide (as counterstain) and 4,6-diamidino-2-phenylindole (DAPI) (for chromosome identification). Images of metaphase preparations were captured by a CCD camera and computer enhanced.

EXAMPLE 4

EXPRESSION VECTORS

The plasmid vector used for expression and selection in eukaryotic cells is based on the pEFBos vector containing the potent promoter (and splice) from the highly expressed elongation factor 1α gene and contains a selectable marker (puroR) driven by the PGK promoter (Mizushima and Nagata, 1990; Visvader *et al.*, 1992). A FLAG epitope tag (Hopp *et al.*, 1988) was incorporated to aid identification of the protein product. The *bcl-w* cDNA was inserted into pEF FLAG-X-PGKpuro, sequenced to confirm the reading frame and transfected by electroporation into FDC-P1 (Dexter *et al.*, 1980), B6.2.16BW2 (Teh *et al.*, 1989) and CH1 (Lynes *et al.*, 1978) cells. Transfectants were selected by culture for 7 days in medium containing 2 μg/ml puromycin and clones subsequently derived from independent pools by limiting dilution.

To detect FLAG-tagged proteins by cytoplasmic immunofluorescence, cells were fixed for 5 min in 80% v/v methanol at -20°C and then permeabilised with 0.3% saponin (Sigma), which was included in all subsequent staining and washing steps. The cells were first incubated with the primary M2 monoclonal antibody (Eastman-Kodak, New Haven, CT) for 40 min on ice, then decorated with fluoroscein-isothiocyanate (FITC)-conjugated goat anti-mouse IgG (1-2 μg/ml; Southern Biotechnology, Birmingham) and analysed by flow cytometry using the FACScan (Becton Dickinson).

EXAMPLE 5

Bcl-w ANTIBODY

Rabbit polyclonal anti-Bcl-w antibodies were raised against the N-terminal peptide Ac-MATPASTPDTRALVC-NH2 [SEQ ID NO:5] (Chiron Mimotopes). KLH-conjugated peptide (100 μg in 0.5 ml of phosphate-buffered saline with an equal volume of Freund's adjuvant) was injected into rabbits and 3, 7 and 14 weeks later the animals were boosted with the same peptide but in Freund's incomplete adjuvant. The rabbit antisera were screened by ELISA against the BSA-conjugated peptide.

EXAMPLE 6

IDENTIFICATION OF A NOVEL *bcl-2*-RELATED GENE

Degenerate PCR primers encoding part of the S2 and S3 regions of the three *bcl-2* homologues known at the time (*bcl-2*, *bcl-x* and *bax*) were used for low-stringency amplification of cDNA templates derived from mRNA of a mouse macrophage cell line and mouse brain (see Example 1). Fragments of the size (159 bp) expected for known Bcl-2 family members were subcloned and screened by sequencing. As anticipated, multiple *bcl-2*, *bcl-x* and *bax* clones were identified, but both RNA sources also yielded clones representing a novel gene. To obtain a full length cDNA, the cloned PCR product was used to probe cDNA libraries from adult mouse brain, spleen and a myeloid cell line. Two classes of cDNA were obtained (Figure 1). The first encoded a polypeptide strikingly similar to Bcl-2, which we termed Bcl-w. The second encoded a much larger polypeptide, which was identical to Bcl-w for the first 144 residues but then diverged markedly. The point of divergence was within the S3 region at a point corresponding to a splice junction in the *bcl-2*, *bcl-x* and *bax* genes. It thus seemed likely that the second class of RNA was produced by alternative splicing of *bcl-w* transcripts.

To resolve this issue, overlapping genomic clones encompassing 22 kb of the *bcl-w* locus (Figure 2) were isolated. The N-terminal portion of the coding region mapped to a 1.3 kb BamHI fragment (subclone c). Its sequence and that of adjacent fragments were determined. Comparison with sequences of the first class of cDNAs revealed that the *bcl-w* gene contained at least 4 closely spaced exons with the coding region split between exon 3 and 4. The unusually large (~ 2.8 kb) 3' untranslated region begins in exon 4. Probes corresponding to either the coding region or the 3' untranslated region of these *bcl-w* cDNAs hybridised to a 3.7 kb polyadenylated RNA of relatively low abundance (Figure 3). The largest *bcl-w* cDNAs are nearly that long.

The second class of cDNAs proved to correspond to chimaeric RNAs produced from *bcl-w* and an adjacent gene (Figure 2). The genomic and cDNA sequences diverge at the 3' end of exon 3, and the 3' portion of the cDNAs derives from an exon approx 9.2 kb downstream (Figure 2).

A probe specific for *rox* of *Drosophila* (see Brand *et al*, 1995) hybridised to abundant mRNAs of 2.8 and 1.8 kb, presumably the *bona fide* (non-chimaeric) transcripts of the *rox* gene. Since cDNA probes corresponding to exon 3 of *bcl-w* detected only the 3.7 kb RNA, the chimaeric *bcl-w/rox* transcripts appear to be of low abundance. The significance of the *bcl-w/rox* transcripts is unclear, and our inability to express detectable levels of the corresponding protein has thus far precluded further analysis.

EXAMPLE 7

THE HUMAN AND MOUSE *bcl-w* GENES ARE HIGHLY HOMOLOGOUS

Human *bcl-w* cDNAs were isolated from a library derived from adult brain mRNA. All six clones corresponded to *bcl-w* cDNA and no chimaeric cDNAs were detected. The coding regions of the human and mouse genes were extremely homologous: 99% identical at the amino acid level and 94% at the nucleotide level. The only amino acid differences were two conservative substitutions: alanine instead of threonine at residue 7 and glutamic acid rather than aspartic acid at residue 124, just upstream of the S3 region. By comparison, the homology of the human and mouse *bcl-2* coding regions is 90% at the amino acid level and 88% at the nucleotide level, and that of *bcl-x* is 97% and 94%.

EXAMPLE 8

THE *bcl-w* GENE ENHANCES CELL SURVIVAL

To allow tests on its function, the *bcl-w* cDNA was inserted into expression vectors and transfected into three haemopoietic cell lines: FDC-P1, an IL-3 dependent myeloid line; B6.2.16BW2, a T hybridoma line; and CH1, a B lymphoma line. To facilitate detection, the recombinant protein included an N-terminal FLAG epitope (Hopp *et al.*, 1988). Several independent pools and clones of drug-resistant cells that stained strongly with an anti-FLAG monoclonal antibody (e.g. Figure 4A) were selected for study. Western blot analysis (Figure 4B) showed that the FLAG-Bcl-w protein had an apparent molecular weight of 25 kD. Rabbit antiserum raised to an N-terminal peptide of Bcl-w detected not only the FLAG-tagged protein but also a protein of ~22 kD, presumably endogenous Bcl-w, that was also apparent in lysates of four untransfected cell lines (Figure 4C). The mobility of the endogenous protein was indistinguishable from that of Bcl-w lacking the FLAG tag transiently expressed in COS cells.

Bcl-w has a hydrophobic region close to its carboxy-terminus and would therefore be expected to be membrane-associated. Confocal microscopy of FDC-P1 cells transfected with a *bcl-w* expression vector and stained with the rabbit polyclonal antibody demonstrated that Bcl-w was located in the cytoplasm and that its distribution closely resembled that of Bcl-2. The cytoplasmic localisation of Bcl-w was confirmed by western blots of nuclear and cytoplasmic protein fractions.

To ascertain whether Bcl-w enhanced or antagonised cell survival, the transfected lines were subjected to various cytotoxic conditions. FDC-P1 cells expressing Bcl-w were notably more robust than the parental cells. Indeed, their survival after either IL-3 deprivation or γ-irradiation was comparable to that of lines over-expressing either Bcl-2 or Bcl-x_L (Figure 5A). Bcl-w also greatly enhanced the survival of the T hybridoma cells exposed to dexamethasone or irradiation (Figure 5B). These results clearly place Bcl-w in the sub-family of Bcl-2-related proteins that inhibits cell death.

Bcl-2 and Bcl-x_L are relatively ineffectual at protecting lymphoid cells against apoptosis induced by triggering the cell surface receptor CD95, also known as Fas or APO-1 (Nagata and Golstein, 1995). That also holds for Bcl-w. CH1 B lymphoma cells expressing levels of Bcl-w sufficient to protect against radiation-induced apoptosis (Figure 5C, right panel) died as rapidly as control cells when incubated with anti-CD95 antibody Jo2 (Figure 5C, left panel). In contrast, the cowpox virus protein CrmA, a potent inhibitor of the ICE cysteine protease (Ray *et al.*, 1992), very effectively blocked apoptosis induced via CD95 (Figure 5C, left panel) but failed to protect the cells from radiation-induced death (Figure 5C, right panel). These results mean that apoptosis is induced by at least two pathways, only one of which involves activation of ICE.

EXAMPLE 9
EXPRESSION PATTERN

Although *bcl-2* and *bcl-x* are both widely expressed, their expression patterns differ significantly (Hockenbery *et al.*, 1991; Krajewski *et al.*, 1994). The expression patterns of *bcl-x* and *bcl-w* were compared by northern blot analysis of polyadenylated RNA. Both genes were expressed in many tissues and each gave highest levels in brain, colon and salivary gland (Table 1). Nevertheless, clear differences emerged upon analysis of a panel of haemopoietic cell lines (Table 2). While *bcl-x* RNA was detected in all 12 T lymphoid lines analysed and a few B lymphoid lines, *bcl-w* expression was rare in T and B lymphoid lines. Transcripts of both survival genes were, however, found in most of the 23 myeloid lines surveyed, which included lines of macrophage, megakaryocytic, erythroid and mast cell origin. Four lines having relatively high levels of *bcl-w* RNA were analysed by western blotting with polyclonal anti-Bcl-w antiserum and each contained the expected 22 kD protein (Figure 4C). These findings establish that the expression pattern of *bcl-w* differs from that of *bcl-x* and raise the possibility that, within the haemopoietic system, *bcl-w* regulates survival in myeloid rather than lymphoid cells.

EXAMPLE 10
LOCALISATION OF THE HUMAN AND MOUSE *bcl-w* GENES

The chromosomal location of *bcl-w* in mice was determined genetically by exploiting an interspecific backcross panel that has been typed for over 2000 loci, well distributed over all the autosomes as well as the X chromosome (Copeland and Jenkins, 1991). Southern blots performed with a *bcl-w* probe on DNA from progeny derived from matings of {(C57BL/6J ¥*Mus spretus*)F_1 ¥ C57BL/6J} mice indicated that *bcl-w* resides in the central region of mouse chromosome 14 linked to surfactant-associated protein 1 (*Sftp1*), T-cell receptor alpha chain (*Tcrα*), and gap junction membrane channel protein alpha-3 (*Gjα3*). At least 134 mice were analysed for every marker, as shown in the segregation analysis (Figure 6) and up to 183 mice

were typed for some pairs of markers. The full data for each pairwise combination of markers was used to calculate recombination frequencies. For each pair of loci, the ratio of the number of mice exhibiting recombinant chromosomes to the number of mice analysed and the most likely gene order are: centromere - *Sftp1* - 14/183 - *Tcrα* - 1/182 - *Bcl-w* - 1/147 - *Gjα3*. The recombination frequencies, expressed in centiMorgans (cM), ± the standard error are: *Sftp1* - 7.7 ± 2.0 - *Tcrα* - 0.6 ± 0.6 - *Bcl-w* - 0.7 ± 0.7 - *Gjα3*.

The central region of mouse chromosome 14 shares regions of homology with human chromosomes 10q, 14q and 13 (summarised in Figure 6). In particular, *Tcrα* has been placed on human 14q11.2 and *Gjα3* on 13. The tight linkage between *Bcl-w*, *Tcrα* and *Gjα3* in the mouse suggested that *Bcl-w* would reside on either human 14q or 13. Fluorescence in situ hybridisation (FISH) analysis using a human cDNA from the coding region and a genomic probe spanning the intron between the coding exons clearly assigned *bcl-w* to human chromosome 14 at q11.2 (Figure 7). Of the 20 metaphases scored for fluorescent signal using the intron probe, 15 showed signal on one or both chromatids of chromosome 14 in the region q11.2-q12 and 90% of the signal was at 14q11.2.

EXAMPLE 11

The nucleotide or corresponding amino acid sequence of human and murine *bcl-w* are shown in Figure 9. The *bcl-w* gene is expressed in many cell types, and amongst the tissues surveyed, the level was highest in brain, colon and salivary gland. A survey of 50 mouse haemopoietic cell lines revealed that *bcl-w* expression was common in cells of several myeloid lineages, including macrophage, megakaryocyte, erythroid and mast cell lines, but rare in either B or T lymphoid lines.

Those skilled in the art will appreciate that the invention described herein is susceptible to variations and modifications other than those specifically described. It is to be understood that the invention includes all such variations and modifications. The invention also includes all of the steps, features, compositions and compounds referred to or indicated in this specification, individually or collectively, and any and all combinations of any two or more of said steps or features.

TABLE 1

TISSUE EXPRESSION OF *bcl-w* AND bcl-x (RNA)

Tissue	*bcl-w*	bcl-x
brain	++	++
colon	++	++
salivary gland	++	++
liver	+	+
heart	+	+/-
stomach	+	+
muscle	+	+/-
testis	+	++[α]
kidney	+/-	+
thymus	+/-	++
lymph node	-	-
bone marrow	?	?
spleen	?	?
placenta	+	++
fetal liver (d13-18)	-	-

[α] larger size transcript

TABLE 2
EXPRESSION PATTERN OF *bcl-w* AND *bcl-x* IN CELL LINES

CELL TYPE	*bcl-w*	*bcl-x*
B lymphoid		
pre-B	1/6	2/6[1]
B	1/4	0/4[1]
	2/10	2/10
T lymphoid		
DN	3/4	4/4
DP	0/7	6/6
SP	1/2	2/2
	4/13	12/12
Myeloid		
macrophage	9/13	10/10
megakaryocyte	2/2	2/2
erythroid	4/5	4/4
mast	1/1	1/1
	21/27	23/23

[1] marginal levels in all but 2 lines indicated

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SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: The Walter and Eliza Hall Institute of Medical Research

(ii) TITLE OF INVENTION: THERAPEUTIC MOLECULES

(iii) NUMBER OF SEQUENCES: 5

(iv) CORRESPONDENCE ADDRESS:
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(C) CITY: MELBOURNE
(D) STATE: VICTORIA
(E) COUNTRY: AUSTRALIA
(F) ZIP: 3000

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER: AU PROVISIONAL
(B) FILING DATE: 27-MAR-1996

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: HUGHES DR, E JOHN L
(C) REFERENCE/DOCKET NUMBER: EJH/EK

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: +61 3 9254 2777
(B) TELEFAX: +61 3 9254 2770

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 33 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

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GCTCTAGAAC TGGGGI(A/C)GI(A/G) TIGTIGCCTT (C/T)TT 33

(2) INFORMATION FOR SEQ ID NO:2:

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(B) TYPE: nucleic acid
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(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

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Asn Trp Gly Arg (Ile/Val) Val Ala Phe Phe
5

(2) INFORMATION FOR SEQ ID NO:3:

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(A) LENGTH: 31 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

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(2) INFORMATION FOR SEQ ID NO:4:

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(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Trp Ile Gln (Asp/Glu) (Asn/Gln) Gly Gly Trp
5

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
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(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

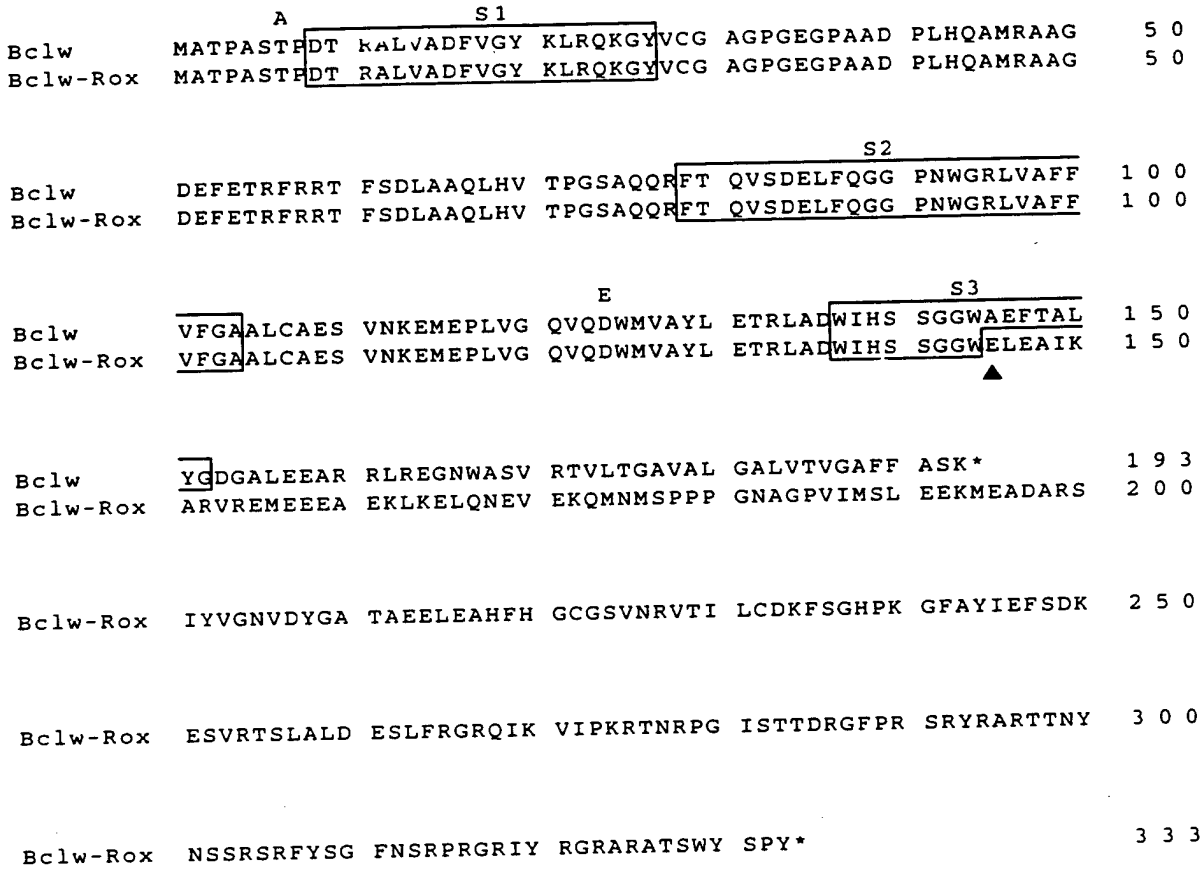
(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

Met Ala Thr Pro Ala Ser Thr Pro Asp Thr Arg Ala Leu Val Cys
5 10 15

DATED this 27th day of March, 1996

The Walter and Eliza Hall Institute
of Medical Research
By Its Patent Attorneys
DAVIES COLLISON CAVE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S1
Bclw MATPASTPDT RALVADFVGY KLRQKGYVCG AGPGEGPAAD PLHQAMRAAG 50
Bclw-Rox MATPASTPDT RALVADFVGY KLRQKGYVCG AGPGEGPAAD PLHQAMRAAG 50
S2
Bclw DEFETRFRRT FSDLAAQLHV TPGSAQQRFT QVSDELFQGG PNWGRLVAFF 100
Bclw-Rox DEFETRFRRT FSDLAAQLHV TPGSAQQRFT QVSDELFQGG PNWGRLVAFF 100
E
S3
Bclw VFGAALCAES VNKEMEPLVG QVQDWMVAYL ETRLADWIHS SGGWAEFTAL 150
Bclw-Rox VFGAALCAES VNKEMEPLVG QVQDWMVAYL ETRLADWIHS SGGWELEAIK 150
Bclw YGDGALEEAR RLREGNWASV RTVLTGAVAL GALVTVGAFF ASK* 193
Bclw-Rox ARVREMEEEA EKLKELQNEV EKQMNMSPPP GNAGPVIMSL EEKMEADARS 200
Bclw-Rox IYVGNVDYGA TAEELEAHFH GCGSVNRVTI LCDKFSGHPK GFAYIEFSDK 250
Bclw-Rox ESVRTSLALD ESLFRGRQIK VIPKRTNRPG ISTTDRGFPR SRYRARTTNY 300
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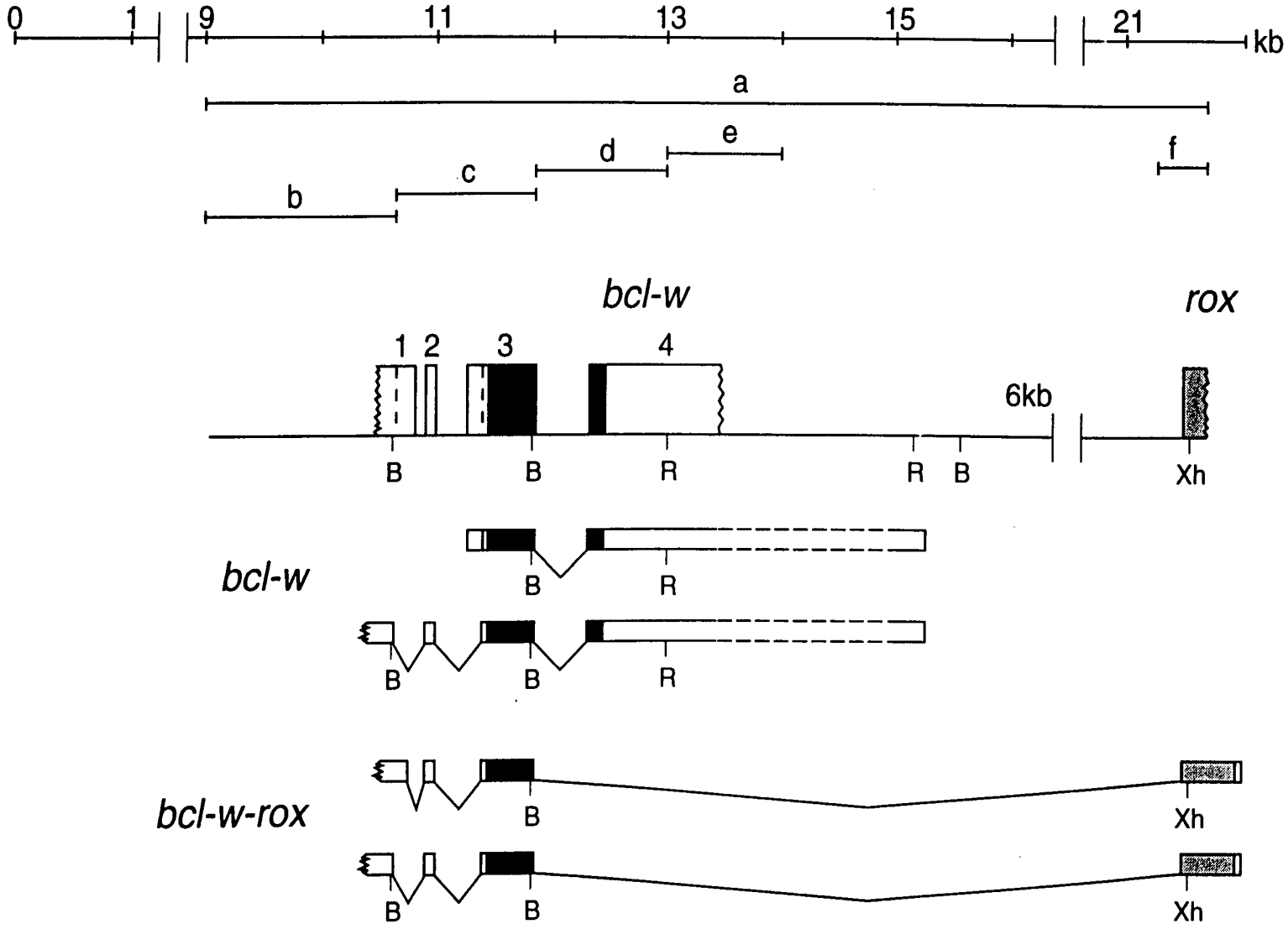
FIGURE 1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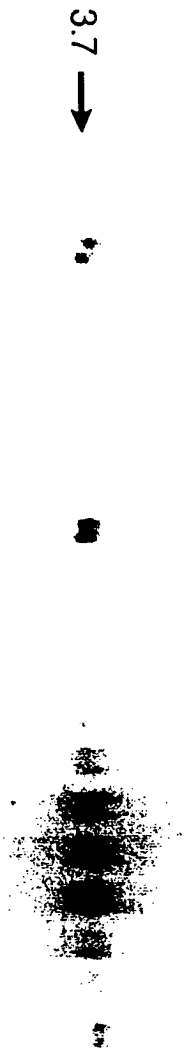
1
9
11
13
15
21
kb
a
b
c
d
e
f
bcl-w
rox
1 2 3 4
6kb
B
B
R
R
B
Xh
bcl-w
B
R
B
B
R
bcl-w-rox
B
Xh
B
B
Xh

FIGURE 2



J774
P388D1
RAW 309Cr
RAW 264.7
3MES/RAF
mθ

LYH7
BAF3
W274
W265
W3BD⁻
FDC-P1
FD/Bcl-2
416B
myeloid

W112.1
W105.7
EL4.1
YAC1
T

W404.1
ABLS8.1
B

FIGURE 3

FIGURE 4

A.
100
0
10^0
10^1
10^2
10^3
10^4
B.
FDC-P1
FD/FLAG-Bcl-2
FD/FLAG-Bcl-x_L
FD/FLAG-Bcl-w
50
34
28
C.
FLAG-Bcl-w
Bcl-w
FD/FLAG-Bcl-w
FDC P1
EL4.1
J774.1
WEHI 112.1
28.5
18

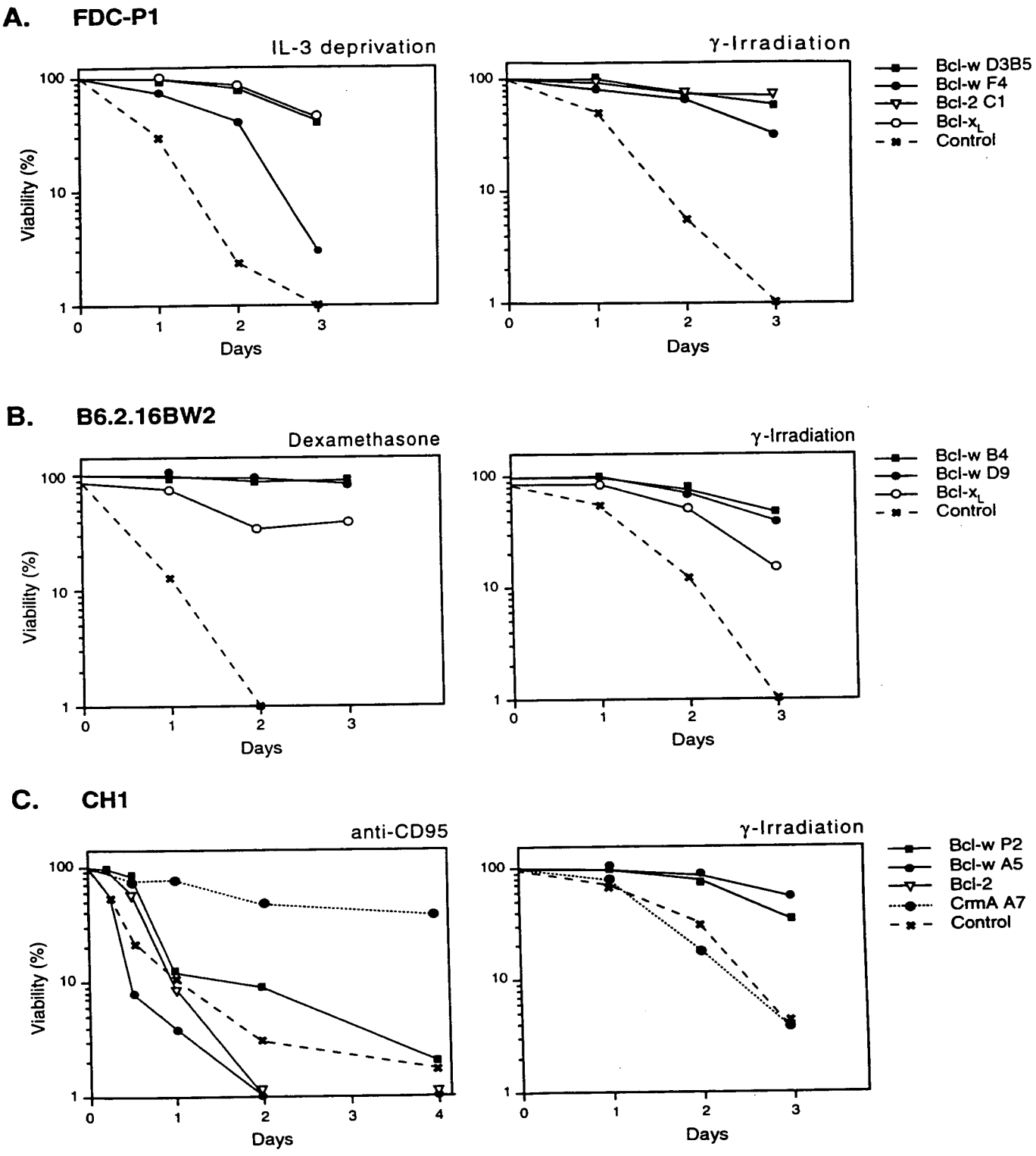
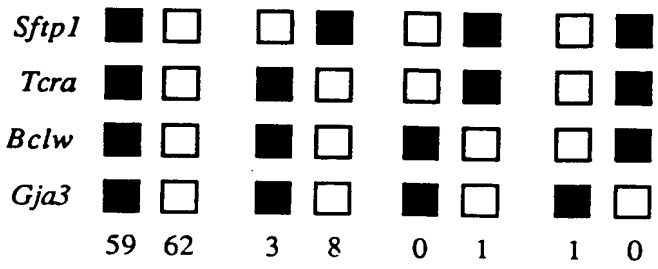

A. FDC-P1
IL-3 deprivation
γ-Irradiation
Viability (%)
Days
Bcl-w D3B5
Bcl-w F4
Bcl-2 C1
Bcl-x$_L$
Control
B. B6.2.16BW2
Dexamethasone
γ-Irradiation
Bcl-w B4
Bcl-w D9
C. CH1
anti-CD95
Bcl-w P2
Bcl-w A5
Bcl-2
CrmA A7

FIGURE 5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Bclw
Gja3
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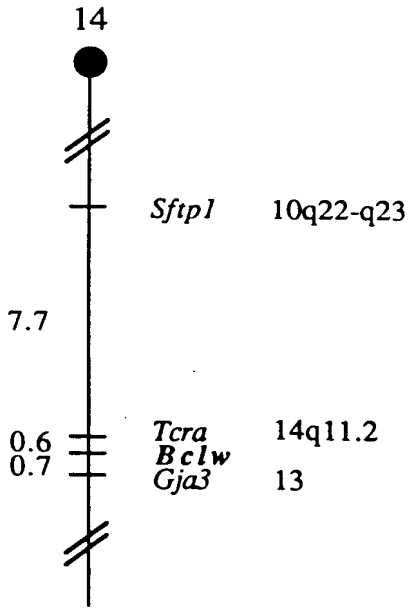
14
Sftp1
10q22-q23
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0.7
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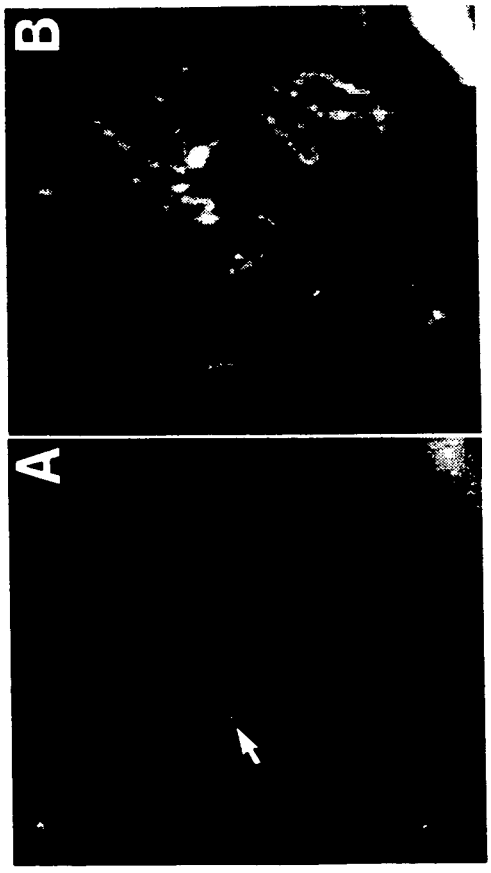
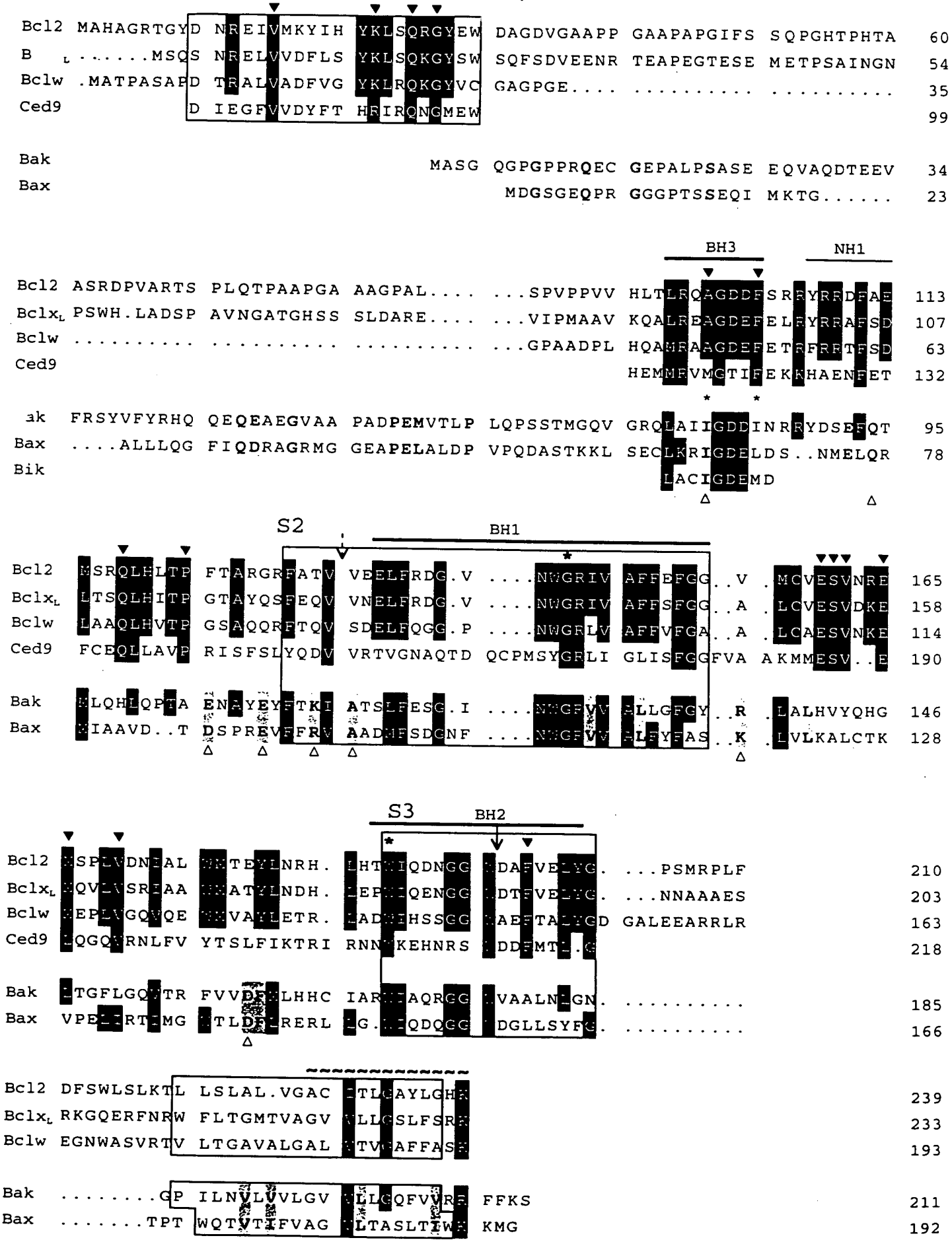
Gja3
14q11.2
13

FIGURE 6

FIGURE 7



RE 8



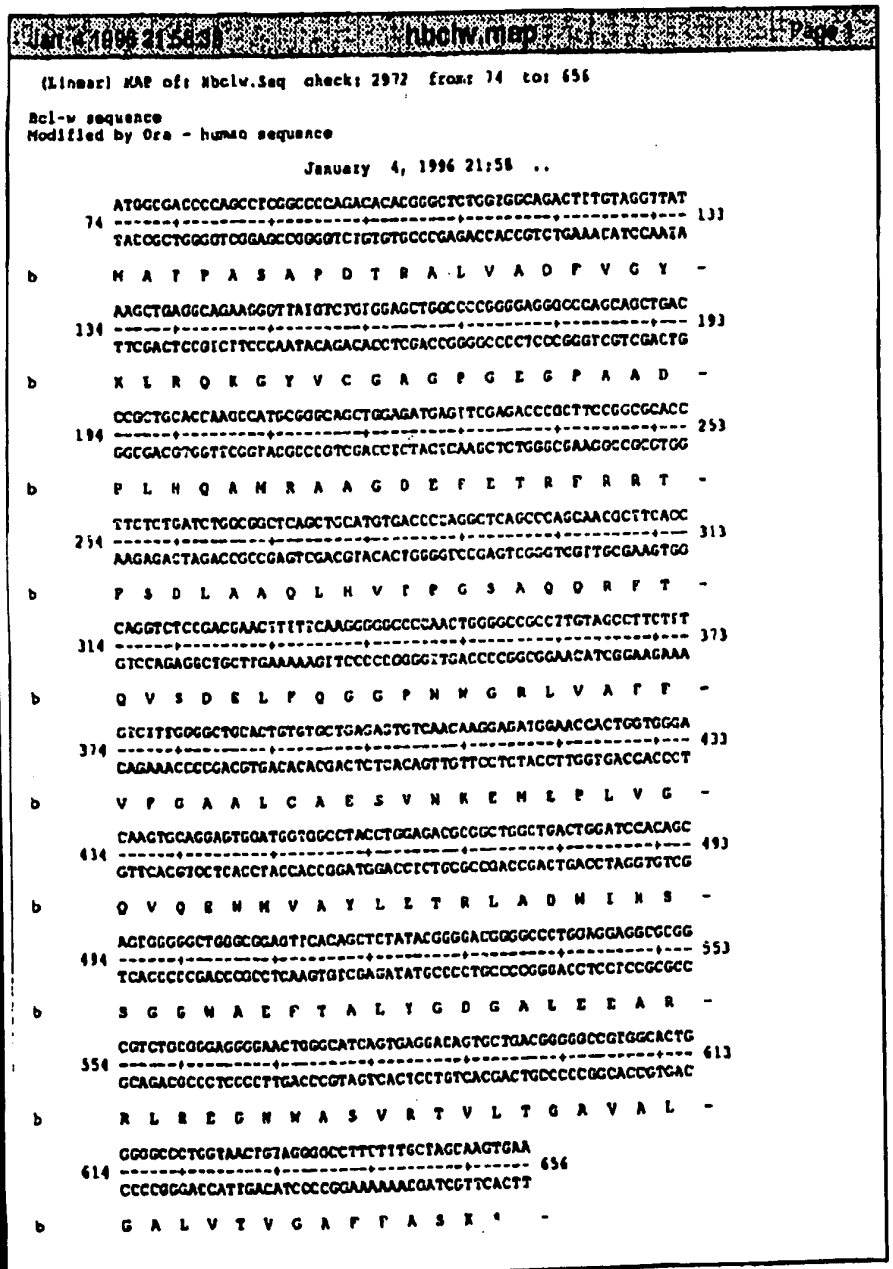


FIGURE 9A

Feb 13 1996 17:28 01 mbclw.map Page 1

(Linear) MAP of: Mbclw.Seq check: 379 from: 227 to: 808

Sequence of 3CLGA (Green) clones from BaT3 library

February 13, 1996 17:28 ..

```
      ATGGCGACCCCAGCCTCAACCCCAGACACACGGGCTCTAGTGGCTGACTTTGTAGGCTAT
  227 ---+---------+---------+---------+---------+---------+------ 286
      TACCGCTGGGGTCGGAGTTGGGGTCTGTGTGCCCGAGATCACCGACTGAAACATCCGATA

b       M  A  T  P  A  S  T  ?  D  T  R  A  L  V  A  D  F  V  G  Y  -

      AAGCTGAGGCAGAAGGGTTATGTCTGTGGAGCTGGCCCTGGGGAAGGCCCAGCCGCCGAC
  287 ---+---------+---------+---------+---------+---------+------ 346
      TTCGACTCCGTCTTCCCAATACAGACACCTCGACCGGGACCCCTTCCGGGTCGGCGGCTG

b       K  L  R  Q  K  G  Y  V  C  G  A  G  P  G  L  G  P  A  A  D  -

      CCGCTGCACCAAGCCATGCGGGCTGCTGGAGACGAGTTTGAGACCCGTTTCCGCCGCACC
  347 ---+---------+---------+---------+---------+---------+------ 406
      GGCGACGTGGTTCGGTACGCCCGACGACCTCTGCTCAAACTCTGGGCAAAGGCGGCGTGG

b       P  L  R  Q  A  M  R  A  A  G  D  E  F  E  T  R  F  R  R  T  -

      TTCTCTGACCTGGCCGCTCAGCTACACGTGACCCCAGGCTCAGCCCAGCAACGCTTCACC
  407 ---+---------+---------+---------+---------+---------+------ 466
      AAGAGACTGGACCGGCGAGTCGATGTGCACTGGGGTCCGAGTCGGGTCGTTGCGAAGTGG

b       F  S  D  L  A  A  Q  L  H  V  T  P  G  S  A  Q  Q  R  F  T  -

      CAGGTTTCCGACGAACTTTTCCAAGGGGCCCTAACTGGGGCCGTCTTGTGGCATTCTTT
  467 ---+---------+---------+---------+---------+---------+------ 526
      GTCCAAAGGCTGCTTGAAAAGGTTCCCCCGGGATTGACCCCGGCAGAACACCGTAAGAAA

b       Q  V  S  D  E  L  F  Q  G  G  P  N  W  G  R  L  V  A  F  F  -

      GTCTTTGGGGCTGCCCCGTGTGCTGAGAGCGTCAACAAAGAAATGGAGCCTTTGGTGGGA
  527 ---+---------+---------+---------+---------+---------+------ 586
      CAGAAACCCCGACGGGACACACGACTCTCACAGTTGTTTCTTTACCTCGGAAACCACCCT

b       V  F  G  A  A  L  C  A  E  S  V  N  K  E  M  E  P  L  V  G  -

      CAAGTGCAGGATTGGATGGTGGCCTACCTGGAGACACGTCTGGCTGACTGGATCCACAGC
  587 ---+---------+---------+---------+---------+---------+------ 646
      GTTCACGTCCTAACCTACCACCGGATGGACCTCTGTGCAGACCGACTGACCTAGGTGTCG

b       Q  V  Q  D  W  M  V  A  Y  L  E  T  R  L  A  D  W  I  H  S  -

      AGTGGCGGCTGGGCGGAGTTCACAGCTCTATACGGGCACGGGGCCCTGGAGGACGCACGG
  647 ---+---------+---------+---------+---------+---------+------ 706
      TCACCGCCGACCCGCCTCAAGTGTCGAGATATGCCCCTGCCCCGGGACCTCCTCCGTGCC

b       S  G  G  W  A  E  F  T  A  L  Y  G  D  G  A  L  E  E  A  R  -

      CGTCTGCGGGAGGGGAACTGGGCATCAGTGAGGACAGTGCTGACGGGGGCCGTGGCACTG
  707 ---+---------+---------+---------+---------+---------+------ 766
      GCAGACGCCCTCCCCTTGACCCGTAGTCACTCCTGTCACGACTGCCCCCGGCACCGTGAC

b       R  L  R  E  G  N  W  A  S  V  R  T  V  L  T  G  A  V  A  L  -

      GGGGCCCTGGTAACTGTAGGGGCCTTTTTTGCTAGCAAGTGA
  767 ---+---------+---------+---------+--------- 808
      CCCCGGGACCATTGACATCCCCGGAAAAAACGATCGTTCACT

b       G  A  L  V  I  V  G  A  F  F  A  S  K  *  -
```

FIGURE 9B